Kinetic Studies of the Reactions Catalyzed by Glucose-6-phosphate Dehydrogenase from *Leuconostoc mesenteroides*: pH Variation of Kinetic Parameters

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The specificity and kinetic parameters of the reactions catalyzed by glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* has been examined under a range of conditions in order to elucidate details about the mechanism of action of this enzyme. The rate of oxidation of glucose 6-phosphate is inhibited by the addition of various organic solvents. However, the low, inherent glucose dehydrogenase activity of this enzyme was stimulated under these conditions, and was further activated by divalent anions that were observed to be inhibitors of the glucose 6-phosphate dehydrogenation. From an examination of the pH variation of the enzyme kinetic parameters two groups on the enzyme that appear to be involved in the binding of the phosphate group of the sugar substrate have been detected. An enzyme catalytic group, probably a carboxylic acid, has been identified that accepts the proton from the hydroxyl group at carbon-1 of the sugar substrate during its oxidation to a lactone. The ionization of a group on the enzyme with a pK of 8.7 resulted in an increase in the maximum velocity of the glucose-6-phosphate dehydrogenase activity of the enzyme as a consequence of a pH-dependent product release step that is no longer rate limiting at high pH. Stabilization of gluconic acid-d-lactone against nonenzymatic hydrolysis by organic solvents has allowed the kinetic parameters of the reverse reaction to be reliably measured for the first time in a narrow pH range.

Glucose-6-phosphate dehydrogenase isolated from yeast has been shown to possess an inherent glucose dehydrogenase activity, but at a greatly reduced level from the glucose-6-phosphate dehydrogenase activity (2, 3). Anderson and Nordlie (4) reported the selective stimulation of the glucose dehydrogenase activity by the anions phosphate, sulfate, and bicarbonate, and the corresponding inhibition of the glucose-6-phosphate dehydrogenase activity. Kinetic studies showed that these anions were competitive inhibitors against glucose 6-phosphate and that there was a single binding site for both the activator and inhibitor functions (5).

Chemical modification studies have implicated several groups as being essential for catalytic activity. Levy *et al.* (6) have identified two essential arginine residues in the *Leuconostoc mesenteroides* enzyme that result in loss of activity when modified by 2,3-butanedione. Inactivation of the enzyme was also observed when a single lysine residue was modified (7). The active-site peptide containing this essential lysine has been isolated and sequenced (8). A lysine was also implicated to function in binding the pyridine nucleotide to the enzyme from *Candida utilis* (9) and essential
tyrosines were also observed in that enzyme by modification studies (10).

This paper describes the effect of organic solvents, anions, and pH on the kinetic parameters of the glucose and glucose-6-phosphate dehydrogenase activities of the enzyme isolated from \textit{L. mesenteroides}.

MATERIALS AND METHODS

Glucose-6-phosphate dehydrogenase isolated from \textit{L. mesenteroides} (D-Glucose 6-phosphate: NADP 1-oxidoreductase, EC 1.1.1.49) was purchased from Sigma as a lyophilized powder and was used without further purification. Sugars, sugar phosphates, and glucose 6-sulfate were also purchased from Sigma. Nucleotides and nucleotide analogs were purchased from P-L Biochemicals. Spectrophotometric-grade organic solvents were from Aldrich.

\textit{Determination of lactones}. The concentration and stability of lactone solutions in various mixed solvents were determined by a modification of the procedure of Hestrin (13). Equal volumes of 2.0 M hydroxylamine hydrochloride and 3.5 M sodium hydroxide were mixed. A 2-ml volume of this solution was added to 1 ml of the lactone solution to be measured. After 90 s, the pH was adjusted to 1.0 with 4 N HCl. A 1-ml volume of a 0.37 M FeCl$_3$ solution in 0.1 N HCl was added and the absorbance was read at 500 nm. Concentrations were determined by comparison to standard curves, after subtraction of a blank.

\textit{Enzyme assays}. Kinetic studies were run in 3.0 ml total volume in 1-cm-path-length cuvettes by measuring absorbance changes at 340 nm with a Gilford Model 250 spectrophotometer. Temperature was maintained at \pm 0.1°C of the stated values with thermospacers and a circulating water bath. Assays were run at 25°C unless otherwise stated. The reaction mixture for measuring the forward reaction contained 50 mM buffer, various levels of sugar and nucleotide substrates, and different levels of anion or organic solvents as stated. The NADP-linked activity of glucose-6-phosphate dehydrogenase was examined in the studies described, except for the nucleotide specificity studies. The ionic strength was maintained constant for a given set of experiments by the addition of potassium acetate. Acetate was shown to have no effect on the rates of the enzyme-catalyzed reaction. The assay conditions for examination of the reverse reaction were 100 mM buffer, 100 mM phosphate, and 0.1 mM NADPH in 40% DMSO. For the pH

\[ \frac{v}{K} = \frac{VA}{K + A} \]  

\[ \log y = \log \left[ \frac{C}{1 + [H^+]K_{s+}} \right] \]  

\[ \log y = \log \left[ \frac{C}{1 + [H^+]K_1 + [H^+]K_2} \right] \]  

\[ \log y = \log \left[ \frac{C}{1 + [H^+]K_1 + [H^+]K_2 + K_3[H^+] + K_4[H^+]^2} \right] \]  

\[ \log y = \log \left[ \frac{C}{1 + [H^+]K_1 + [H^+]K_2 + K_3[H^+] + K_4[H^+]^2} \right] \]  

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\*Abbreviations used: DMSO, dimethylsulfoxide; DMF, \textit{N,N}-dimethylformamide; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; G6P, glucose 6-phosphate.
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\[
\log y = \log \left( \frac{L_O + H_I \times K_I / [H^+]^I}{1 + K_I / [H^+]^I} \right) \quad [5]
\]

\[
v = \frac{V_A}{K(1 + I/K_a) + A} \quad [6]
\]

The standard deviations of the kinetic parameters fitted to the above equations are ±5% or less of the fitted value. The standard deviations of the fitted pK values are ±0.1 pH units or smaller.

RESULTS

Effects of organic solvents. Glucose-6-phosphate dehydrogenase from L. mesenteroides was observed to possess an inherent glucose dehydrogenase activity that was about 0.3% that of its glucose-6-phosphate dehydrogenase activity at pH 8.0 when the rates were examined in the presence of phosphate buffer. Addition of organic solvents to these reaction mixtures caused a decrease in the maximum velocity of the enzyme-catalyzed oxidation of glucose 6-phosphate, and an increase in the maximum velocity of the glucose dehydrogenase reaction. These effects were observed with several different organic solvents, but the rates have been examined in detail only with N,N-dimethylformamide and with dimethylsulfoxide (Fig. 1). In the presence of organic solvents the V/K values for the sugars were altered, reflecting an increased affinity of the enzyme for the sugar and sugar phosphate substrates (Table I). The maximum velocity of the reaction increased in the presence of higher DMSO levels with glucose as the substrate, while V_{max} of the glucose 6-phosphate oxidation decreased. The rate of oxidation of glucose 6-sulfate was increased similarly to that of glucose by the addition of organic solvents (Table I).

Substrate specificity. The specificity of glucose-6-phosphate dehydrogenase for its carbohydrate substrate was examined at pH 8, with NADP present at saturating levels (0.4 mM). The results in Table II indicate that replacement of the 2-hydroxyl group of glucose 6-phosphate or replacement of the phosphate group on carbon-6 by sulfate resulted in a decrease of two orders of magnitude in the maximum velocity. These changes also gave a decrease of four to five orders of magnitude in V/K for 2-amino-D-glucose 6-phosphate and glucose 6-sulfate, respectively.

When the reaction was run in 40% DMSO in the presence of 50 mM phosphate, a similar change in the maximum velocity was seen when the 2-hydroxyl group of glucose was replaced with an amino group (Table II). When the stereochemistry about carbons-3 or -4 was altered the resulting sugars showed virtually no substrate activity at concentrations up to 200 mM. Modification at carbon-6 of glucose had only a small effect on V/K for sugars, and the maximum velocity varied by a factor of 10 from 6-deoxy-D-glucose (−CH₂OH replaced by −CH₃) to D-xylOSe (−CH₂OH replaced by −H).

In addition to NAD and NADP the enzyme from L. mesenteroides was observed to utilize several other nucleotide cofactor analogs as substrates, when examined in 40% DMSO, with 50 mM phosphate and 400 mM glucose (five times K_m). Alterations at carbon-6 of the adenine ring (deamino-NAD) or at the carbonyl group of the nicotinamide moiety (3-acetylpyridine- or 3-
TABLE I
EFFECT OF DMSO ON KINETIC PARAMETERS

<table>
<thead>
<tr>
<th>Substrate</th>
<th>DMSO (%)</th>
<th>Phosphate (mM)</th>
<th>$V_{\text{max}} \times 10^2$ (pmol ml$^{-1}$ min$^{-1}$)</th>
<th>$V/K_{\text{max}} \times 10^3$ (min$^{-1}$)</th>
<th>$K_{\text{sugar}}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>20</td>
<td>50</td>
<td>0.21</td>
<td>0.006</td>
<td>340</td>
</tr>
<tr>
<td>Glucose</td>
<td>40</td>
<td>50</td>
<td>0.76</td>
<td>0.096</td>
<td>80</td>
</tr>
<tr>
<td>Glucose</td>
<td>40</td>
<td>0</td>
<td>0.55</td>
<td>0.011</td>
<td>530</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>0</td>
<td>0</td>
<td>4.0</td>
<td>370</td>
<td>0.11</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>0</td>
<td>50</td>
<td>4.0</td>
<td>130</td>
<td>0.30</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>10</td>
<td>50</td>
<td>2.3</td>
<td>230</td>
<td>0.11</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>30</td>
<td>50</td>
<td>0.2</td>
<td>110</td>
<td>0.015</td>
</tr>
<tr>
<td>Glucose 6-sulfate</td>
<td>0</td>
<td>0</td>
<td>0.05</td>
<td>0.005</td>
<td>100</td>
</tr>
<tr>
<td>Glucose 6-sulfate</td>
<td>20</td>
<td>0</td>
<td>0.03</td>
<td>0.023</td>
<td>170</td>
</tr>
<tr>
<td>Glucose 6-sulfate</td>
<td>40</td>
<td>0</td>
<td>0.76</td>
<td>0.80</td>
<td>94</td>
</tr>
</tbody>
</table>

*The assay conditions for these experiments were 50 mM Hepes buffer (pH 8.0), 0.4 mM NADP, phosphate and DMSO at the levels stated, and acetate added to maintain constant ionic strength. The kinetic parameters were determined by least squares fits of the data as described under Materials and Methods.

pyridinealdehyde-NAD) caused a decrease in both the maximum velocity (up to a factor of 30) and the $V/K$ for nucleotides (up to a factor of 200), as shown in Table III.

**Effect of anions.** The effect of anions on the reaction rates of the enzyme from *L. mesenteroides* were examined at pH 8 in assay mixtures containing 50 mM Hepes buffer, 0.4 mM NADP, 40–400 mM glucose 6-phosphate, and varying levels of anion inhibitors. Carbonate, phosphate, sulfate, and sulfite were all found to be competitive inhibitors against glucose 6-phosphate, with $K_{i}$ values of about 30 mM for phos-

TABLE II
SUGAR SUBSTRATE SPECIFICITY

<table>
<thead>
<tr>
<th>Sugar</th>
<th>$V_{\text{max}} \times 10^2$ (pmol ml$^{-1}$ min$^{-1}$)</th>
<th>$V/K_{\text{sugar}} \times 10^3$ (min$^{-1}$)</th>
<th>$K_{\text{sugar}}$ (mM)</th>
<th>Modification at carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-Glucose 6-phosphate</td>
<td>4.2</td>
<td>370</td>
<td>0.11</td>
<td>—</td>
</tr>
<tr>
<td>2-Amino-d-glucose 6-phosphate</td>
<td>0.03</td>
<td>0.03</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>d-Glucose 6-sulfate</td>
<td>0.05</td>
<td>0.005</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>d-Glucose</td>
<td>0.06</td>
<td>0.096</td>
<td>90</td>
<td>—</td>
</tr>
<tr>
<td>2-Amino-d-glucose</td>
<td>0.004</td>
<td>0.0005</td>
<td>78</td>
<td>2</td>
</tr>
<tr>
<td>d-Allose</td>
<td>&lt;0.001</td>
<td>—</td>
<td>—</td>
<td>3</td>
</tr>
<tr>
<td>d-Galactose</td>
<td>&lt;0.001</td>
<td>—</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>6-Deoxy-d-glucose</td>
<td>1.9</td>
<td>0.019</td>
<td>1000</td>
<td>6</td>
</tr>
<tr>
<td>d-Xylose</td>
<td>0.18</td>
<td>0.019</td>
<td>90</td>
<td>6</td>
</tr>
<tr>
<td>d-Glucose 6-sulfate</td>
<td>0.76</td>
<td>0.080</td>
<td>94</td>
<td>6</td>
</tr>
</tbody>
</table>

* Assay conditions for the glucose-6-phosphate dehydrogenase studies (first three sugars) were 50 mM Hepes buffer (pH 8.0), 0.4 mM NADP, and no added phosphate or DMSO. For the glucose dehydrogenase studies (remaining sugars) the conditions were the same except 50 mM phosphate was added and the reactions were measured in 40% DMSO. Constant ionic strength was maintained by the addition of acetate and least squares fits of the data were performed as described under Materials and Methods.
TABLE III
NUCLEOTIDE SUBSTRATE SPECIFICITY

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>( V_{\text{max}} \times 10^2 ) (( \mu \text{mol m}^{-1} \text{min}^{-1} ))</th>
<th>( \frac{V}{K_{\text{m}} \times 10^5} ) (min(^{-1} ))</th>
<th>( K_{\text{m}} ) (mM)</th>
<th>Modification at carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD</td>
<td>0.55</td>
<td>18.0</td>
<td>0.80</td>
<td>—</td>
</tr>
<tr>
<td>NADP</td>
<td>0.37</td>
<td>690.0</td>
<td>0.005</td>
<td>2' (Ribose)</td>
</tr>
<tr>
<td>3-Acetylpyridine-NAD</td>
<td>0.08</td>
<td>0.2</td>
<td>4.4</td>
<td>Carbonyl (Nicotinamide)</td>
</tr>
<tr>
<td>3-Pyridinealdehyde-NAD</td>
<td>0.02</td>
<td>0.08</td>
<td>1.2</td>
<td>Carbonyl (Nicotinamide)</td>
</tr>
<tr>
<td>Deamino-NAD</td>
<td>0.13</td>
<td>0.2</td>
<td>6.5</td>
<td>6 (Adenine)</td>
</tr>
</tbody>
</table>

*The assay conditions were 50 mM Hepes buffer (pH 8.0), 50 mM phosphate, and 400 mM glucose in 40% DMSO. Constant ionic strength was maintained by added acetate and the kinetic parameters were determined as described under Materials and Methods.

phosphate and sulfate and 165 mM for carbonate at pH 8.0. These same anions were found to be activators of the glucose dehydrogenase reaction, either in the presence or absence of organic solvents, with phosphate, sulfate, and sulfite having similar activation constants. Nitrate and nitrite were also examined, but these anions did not inhibit or activate glucose-6-phosphate dehydrogenase.

Reverse reaction. While gluconic acid-\( \delta \)-lactone, the product of the forward reaction with glucose as the substrate, is fairly unstable, the rate of hydrolysis of the lactone can be minimized in a narrow pH range at increasing concentrations of DMSO. The lactone was found to be stable for several hours in 40% DMSO at low pH, with the half-time for hydrolysis decreasing to about 75 min at pH 6.6.4 Studies of the reverse reaction were further hampered, however, by the instability of the reduced nucleotides (NADH and NADPH) below pH 7. The kinetic parameters for the reverse reaction were determined in the pH range from 5.5 to 7.0, after subtraction of the nonenzymatic hydrolysis rate of the substrates. At pH 6.5, the maximum velocity was 0.024 \( \mu \text{mol m}^{-1} \text{min}^{-1} \), \( \frac{V}{K_{\text{m}}} \) for the lactone was \( 1.9 \times 10^{-4} \) min\(^{-1} \), and the Michaelis constant for the lactone was 125 mM. \( \frac{V}{K} \) for the lactone seemed to be independent of pH in the narrow range in which it was examined, while the maximum velocity decreased at the higher and lower pH values in this pH range. Attempts to expand this pH range to determine if

![Fig. 2. pH dependence of glucose-6-phosphate dehydrogenase activity.](image-url)
the catalytic group observed in the forward direction showed reciprocal protonation behavior, i.e., if deprotonation caused loss of activity in the reverse reaction, were unsuccessful. The ratio of the maximum velocity of the reaction in the forward direction to that in the reverse direction was determined to be about 25 at pH 6.5.

pH variation of kinetic parameters. The dependence of the kinetic parameters of the glucose-6-phosphate dehydrogenase reaction on pH was examined at 0.4 mM NADP and varying levels of glucose 6-phosphate. $K_{\text{NADP}}$ was determined at the extremes of the pH range examined to ensure that NADP remained saturating. $V_{\text{max}}$ and $V/K$ data were plotted as described under Materials and Methods to obtain pK values. The $V/K_{\text{G6P}}$ profile (Fig. 2B) was pH independent in the pH region from 6.5 to 8.5, but decreased sharply on both the acidic and basic sides of that range to limiting slopes of 2 and -2, respectively. At high pH, the best fit to the data was obtained by assuming that the ionization of two groups caused the loss of activity in the free enzyme, with pK values of 9.3 and 10.3. At low pH, protonation of two groups also caused decreased activity, with fitted pK values of 4.5 and 5.5. The pK observed at 5.5 is that of the substrate glucose 6-phosphate and not that of a group on the enzyme. Only two pK values were observed in the $V_{\text{max}}$ profile, at pH 6.8 and 6.7 (Fig. 2A). However, protonation of these groups in the enzyme-substrate complex resulted in enzyme forms which were still active, but at a lower rate.

Similar results were obtained for the glucose dehydrogenase activity at saturating levels of NADP in the presence of 50 mM phosphate and 40% DMSO. The $V/K$ profile for glucose (Fig. 3B) had the same shape as the $V/K_{\text{G6P}}$ profile (Fig. 2B), except that the entire profile was displaced to the basic side. The decrease in activity on the acidic side was best fit to two ionizable groups which were protonated with pK values of 6.0 and 6.8. The pK observed at 6.8 is due to protonation of the phosphate which is present as an activator in the glucose dehydrogenase reaction. On the basic side, the decrease in activity appears to be a result of the ionization of a single group with a pK of 9.9. The second group observed on the basic side of the $V/K$ profile for glucose 6-phosphate was presumably displaced too far to be observed. The $V_{\text{max}}$ profile was essentially pH independent above pH 6, and decreased at low pH toward a limiting slope of 2 (Fig. 3A). The decrease on the acidic side was best fit by the protonation of two groups with pK values of about 5.5. When the pH dependence of the glucose dehydrogenase activity was re-examined in the presence of saturating (3 mM) NAD, the $V_{\text{max}}$ and $V/K_{\text{glucose}}$ pH profiles that were obtained were virtually identical to those obtained with saturating NADP (Fig. 3).

Since the acidic side of the $V/K$ profiles for glucose and glucose 6-phosphate reflected the protonation of a phosphate group on the activator or substrate (phosphoric acid or glucose 6-phosphate), then only a single pK should be observed on the acidic side of the pH profile if the pH study is repeated without a phosphate group present. This was observed when the $V/K$ profile for glucose 6-sulfate was examined in 40% DMSO in the absence of phosphate (Fig. 4, curve A). The pH profile was fitted with a pK value of 6.1. When the pH study
FIG. 4. Effect of DMSO on the $V/K_{\text{glucose-6-phosphate}}$ pH profile. The individual data points were determined as in Fig. 2, with 0.4 mM NADP, varying levels of glucose 6-sulfate, and no phosphate present. The curves through the data are fits to Eq. [2]. (A) DMSO = 40%; (B) DMSO = 0%.

was repeated in the absence of DMSO (Fig. 4, curve B) protonation of the group on the enzyme became more difficult, and the $pK$ value decreased to about 4.8.

**pH dependence of anion binding.** The pH dependence of the competitive inhibition constant, $K_{in}$, of several anions against glucose 6-phosphate was examined to establish the role of the groups on the enzyme whose $pK$ values were observed on the basic side of the $V/K$ profiles. Phosphate, sulfate, and carbonate gave similar $pK_{in}$ profiles, with the ionization of a group on the enzyme with a $pK$ value of 9.2–9.6 (Fig. 5) causing a loss of binding. The $pK_{in}$ profile for carbonate (Fig. 5, curve C) also reflected a $pK$ on the acidic side at 9.0 corresponding to the protonation of the carbonate anion.

**DISCUSSION**

Glucose-6-phosphate dehydrogenase is fairly nonspecific for its nucleotide substrate when the reaction is run in the absence of organic solvents. The enzyme will utilize either of the common pyridine nucleotide cofactors NAD or NADP (18), and also shows fairly high levels of activity with modified pyridine nucleotides. The enzyme shows a higher degree of specificity for its sugar substrate. Salas *et al.* (3) reported that 2- and 3-deoxyglucose 6-phosphate, and galactose 6-phosphate, gave rates that were two orders of magnitude slower than the natural substrate when examined at fixed concentrations with yeast glucose-6-phosphate dehydrogenase. 2-Amino-D-glucose 6-phosphate is a much poorer substrate for the enzyme (Table II), even though the amino group is greater than 40% unprotonated at pH 8.0. The large decrease in $V/K$ indicates that the hydroxyl groups at carbons-2 and-3 play an important role in the substrate binding to the enzyme. Chemical modification studies have implicated the $\varepsilon$-amino group of a lysine as being essential for activity in the enzyme from *L. mesenteroides* and *C. utilis*. An essential tyrosine was also found in the enzyme isolated from *C. utilis*. These functional groups could be interacting with the sugar substrate.

Changes at carbon-6, either replacing the phosphate group with sulfate or removing it completely, also cause a sharp decrease in activity. However, when the sugar substrate specificity was examined in 40% DMSO with 50 mM phosphate present, substitutions at carbon-6 did not have a drastic effect on substrate activity. Glucose (−CH$_2$OH attached to carbon-5), 6-de-
oxy-D-glucose (−CH₃), glucose 6-sulfate (−CH₂OSO₃), and D-xylose (−H) were all good substrates for the enzyme, with surprisingly little variation in kinetic parameters. Substitution at carbon-2 (−NH₂) resulted in a decreased maximum velocity, and the epimers at carbons-3 and -4 (D-allose and D-galactose) showed negligible substrate activity in the presence of organic solvents.

Anions such as phosphate, carbonate, and sulfate apparently activate the glucose dehydrogenase reaction by occupying the subsite for the phosphate moiety of glucose 6-phosphate. These activators must be in the dianion form to bind to the enzyme. Nitrate and nitrite, which are monoanions, did not activate the glucose dehydrogenase reaction, nor did they inhibit the glucose-6-phosphate dehydrogenase reaction. Also, protonation of phosphate (pK₂) and of carbonate to bicarbonate caused a loss in the ability to activate the enzyme. The enzyme showed no specificity in the binding of divalent anions, with all the inhibitors tested having similar Kᵢ values (when carbonate was corrected for the concentration that was in the dianion form).

The addition of organic solvents had a pronounced effect on the activity of the enzyme. The effects which were observed do not appear to be a result of specific binding of organic solvents to the enzyme since these effects were seen with several different organic solvents, although the addition of DMSO resulted in the largest changes in the kinetic parameters. Organic solvents seem to exert their effect by increasing the affinity of the enzyme for its substrates. In the case of a negatively charged substrate like glucose 6-phosphate this is not difficult to rationalize, since the solvent is becoming less polar and the partitioning between free and enzyme-bound sugar phosphate would be expected to be shifted toward the enzyme-bound form. For neutral sugars like glucose, the additional binding attraction may come from the altered conformation of the enzyme active site in response to the changing solvent composition. The presence of phosphate or other small dianionic species probably induces further changes in the conformation of the active site to accommodate the binding of glucose.

Identification of catalytic and binding groups. For the reaction run in the absence of organic solvents, four groups whose state of ionization affected enzyme activity were identified from the V/K profile for glucose 6-phosphate. Protonation of glucose 6-phosphate (pK = 5.5) resulted in a loss of activity (Fig. 2B), confirming that the sugar phosphate substrate must be in the dianion form to bind to the enzyme. At lower pH, the protonation of a group on the enzyme with a pK value of 4.5 resulted in a form of the enzyme which was inactive. This group probably is the base on the enzyme which accepts the proton from the hydroxyl group at carbon-1 of glucose 6-phosphate during its oxidation to the lactone. Protonation of this base would destroy activity. When the reaction was run in 40% DMSO with glucose as the substrate, the pK of this group shifted from 4.5 to 6.0 (Fig. 3B). This increase in pK is the characteristic response of a neutral acid group such as a carboxylate to changes in solvent composition. The same behavior was observed when the reaction was run with glucose 6-sulfate as the substrate. In the absence of organic solvents a pK was observed in the V/K profile for glucose 6-sulfate at about 4.8, which increased to 6.1 in the presence of 40% DMSO (Fig. 4). Since glucose 6-sulfate does not have a pK in this pH range, this substrate remains in the monoanion form, accounting for the very low V/K values observed, and confirming the assignment of the pK value in Fig. 2B to glucose 6-phosphate. In the absence of a substrate pK, the V/K pH profile for glucose 6-sulfate showed only the protonation of the acid-base catalytic group on the enzyme at low pH.

*Titration of glucose 6-phosphate gave a value of 6.0 for the pK₂ of the phosphate group. The difference in pK value that was observed for glucose 6-phosphate in these enzyme pH profile studies is probably a result of the difficulty of separating two overlapping ionizations that differ by only one pH unit.
The ionization of two groups at high pH also affected enzyme activity. These groups were observed in the $V/K$ profile for glucose 6-phosphate with $pK$ values of 9.3 and 10.3. A previous pH study of the NAD-linked activity (11) had observed a group on the enzyme with a $pK$ value of 8.9. Kuby and Roy (12) also observed the ionization of a group at pH 9.1 in the enzyme isolated from brewer's yeast that they assigned to a lysine. In the $V/K$ profile for glucose (Fig. 3B) only a single $pK$ was observed at high pH, presumably because of the displacement to higher pH caused by the presence of phosphate. Since the $V/K$ profile for glucose was run in the presence of high levels of phosphate, the $pK$ which was not observed may have been from the group on the enzyme which binds the phosphate moiety of glucose 6-phosphate.

The pH variation of the inhibition constant, $K_i$, was examined for several anions that were competitive inhibitors against glucose 6-phosphate. Any $pK$ values which are observed in these $K_i$ profiles would reflect groups which must be in the correct ionization state to bind the anionic portion of the sugar phosphate substrate. Sulfate and phosphate had similar $pK_i$ profiles, with a single $pK$ on the basic side with a $pK$ of 9.2–9.6 (Fig. 5). This confirms the role of one of the basic groups observed in the $V/K$ profile for glucose 6-phosphate. Ionization of this group would cause loss of enzyme activity, since the ability of this group to act as a hydrogen bond donor to the phosphate moiety of glucose 6-phosphate would be eliminated. The $pK_i$ profile for carbonate also reflected this loss of binding at high pH but, in addition, showed a loss of binding caused by the protonation of carbonate itself, confirming that the sugar phosphate or the anion inhibitor must be in the dianion form in order to bind to the enzyme. The $K_i$ values for these anions are in the range 20–30 mM at their pH maxima (Fig. 5), indicating that the enzyme has a similar affinity at the phosphate binding site for $CO_3^{2-}$, $SO_4^{2-}$, and $HPO_4^{2-}$.

The two groups whose state of ionization affected the maximum velocity of the glucose-6-phosphate dehydrogenase reaction both appear to result in only partial changes in enzyme activity. In both cases the protonated form of the enzyme was less active, but still retained some activity. The protonation observed with a $pK$ of 8.7 caused a 40% decrease in the maximum velocity, while the protonation of a group with a $pK$ of 6.3 caused a decrease in activity of a factor of 100. Alternately, the $pK$ observed at 6.3 could be explained by assuming that the loss of activity at low pH was the result of a change in the overall charge of the protein. This would result in a linear decrease in activity below the $pK$ value, but would not require an integral slope. The stability of the enzyme is greatly diminished below pH 4, precluding the acquisition of reliable data that could distinguish between these alternate interpretations. Kuby and Roy (12) reported a biphasic maximum velocity profile that they attributed to a second pathway for product formation at high pH. The ionization of the group on the enzyme with a $pK$ of 8.7 caused an increase in the overall rate of the reaction because a pH-dependent step (or steps) is no longer limiting the reaction rate. Because this change in rate is observed only at high substrate concentrations ($V_{\text{max}}$ conditions) and not at low substrate ($V/K$ conditions) this pH-dependent step must occur after the steps involved in bond making and breaking. All the steps in the mechanism before these chemical events appear in both the $V_{\text{max}}$ and $V/K$ expressions. The $pK$ value of 8.7 must therefore be assigned to a step in the mechanism involved with product release or an enzyme conformation change associated with release of product. The maximum velocity of the reaction below pH 8.7 (with product release partially rate limiting) was determined to be 0.043 µmol ml$^{-1}$ min$^{-1}$. With product release not limiting the rate above pH 8.7 the maximum velocity increased to 0.082 µmol ml$^{-1}$ min$^{-1}$. The product release rate at neutral pH can be calculated by the difference between the reciprocals of the rates above and below pH 8.7, assuming that the other step or
steps that are rate limiting are not pH dependent in the pH range.

\[
\frac{1}{V_{\text{max}}(<\text{pH } 8.7)} = \frac{1}{V_{\text{max}}(>\text{pH } 8.7)}
\]

This change in rate-limiting steps is not observed for the reactions run in the presence of organic solvents. This is probably because the release of product becomes rate limiting at all pH values in the presence of organic solvents (Viola and Cleland, unpublished results).

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